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# Enzymatic keratin hydrolysis: Dynamic modelling, parameter estimation and validation

Alistair Rodman<sup>a</sup>, Francesco Falco<sup>b</sup>, Dimitrios I. Gerogiorgis<sup>a\*</sup>, Krist V. Gernaey<sup>b</sup>

<sup>a</sup> *School of Engineering (IMP), University of Edinburgh, Edinburgh, EH9 3FB, UK*

<sup>b</sup> *DTU Chemical Engineering, Technical University of Denmark, 2800 Kgs. Lyngby, DK*

*D.Gerogiorgis@ed.ac.uk*

## Abstract

Keratin rich waste material is an abundant by-product from the agroindustry, particularly the meat and poultry industries: skin remains, bristle, animal hair, horns and hooves, feathers, etc. This waste may not be incinerated due to environmental concerns, so producers seek waste valorization by upcycling this non-biodegradable by-product by depolymerization to extract soluble proteins, which can be used as animal feed supplements. This can be performed thermally, however high temperature processing destroys amino acids which are necessary in the product and are costly to later supplement. A novel two-stage enzymatic de-polymerization process for keratin is being investigated. The first stage involves growing the microbial keratinases on a substrate sample, and is optimized for maximal enzyme production. The second stage uses the keratinases in a bioreactor optimized for substrate hydrolysis. The enzymatic hydrolysis mechanism for keratin is not well documented rendering current the current industrial application limited. This paper presents lab scale experimental results from the second (hydrolysis) stage using a keratinolytic enzymatic cocktail with the filamentous bacterium *Amycolatopsis keratiniphila* D2 (DSM 44409). Dynamic state data for the product (protein) and substrate (keratin) concentrations following varying substrate loading has been used to construct the first reduced order model for the enzymatic hydrolysis of waste keratin. Potential model applications include to dynamically optimize this second process stage by computing optimal dosage strategies (keratin deposit intervals and volume) to minimize processing time and cost to dispose or repurpose the biochemical waste.

**Keywords:** keratin, enzymatic hydrolysis, dynamic modelling, parameter estimation.

## 1. Introduction

### 1.1. Background

Keratin rich waste material is an abundant by-product from agroindustrial activities, particularly the meat and poultry industries (Daroit and Brandelli, 2014): skin remains, bristle, animal hair, horns and hooves, feathers, etc. It is estimated that five million tonnes per year of keratin waste is produced in these industries (Brehu and Spiridon, 2011), which is classified as a low-risk animal by-product. This constitutes the third most abundant renewable polymeric material present in nature after cellulose and chitin. This solid residue is not suitable for human consumption and must be treated for safe disposal, providing financial and environmental incentives for process development.

Recently, producers seek waste valorization by upcycling this non-biodegradable by-product by depolymerization to extract soluble proteins from the residual biomass to produce a saleable by-product, for example as an animal feed supplement. The aquaculture industry is one of the fastest growing sectors in food production, accounting for approximately half of the seafood consumed in the world. The availability of risk-free, easily accessible and economical feed ingredients for sustainable aquaculture production plays a key role in global food security; proteins obtained from the biodegradation of keratin could replace a significant fraction of the fish meal used in aquaculture feed formulation. Fish meal constitutes one of the main ingredients of fish feed and represents about 40% of its total weight (Fang et al., 2013), suggesting vast potential demand for such a product, under given purity and food safety specifications.

### *1.2. Enzymatic keratin bioprocessing*

Traditionally keratin hydrolysis has been performed thermally (Jeske et al., 1976; Orzeszko and Sutarzewicz, 1979), however high temperature processing destroys amino acids necessary for the product to be used as animal feed and are costly to later supplement. Keratins are fibrous structural proteins containing many disulfide bonds in their primary structure. Based on their secondary structure, keratins, are divided into  $\alpha$ - and  $\beta$ -keratin. The  $\alpha$ -type (hard keratin) has a higher cysteine content which allows the formation of a larger number of disulfide bridges between cross-linking protein chains, rendering them water-insoluble and resistant to lysis by conventional proteolytic enzymes. Select bacteria, actinomycetes and keratinophilic fungi, have been found capable of synthesizing microbial keratinases (enzymes which hydrolyse keratins) when in an environment that the only source of carbon, nitrogen, sulphur and energy is from keratin biomass. Therefore, biodegradation using keratinolytic bacteria is an attractive way of converting keratinic waste into products of practical industrial value (Al-Musallam et al., 2003). This can include acting as a fish meal replacement in feeds for the aquaculture industry, where the product has an improved amino acid profile compared to thermal keratin processing (Korniłowicz-Kowalska and Bohacz, 2011).

Therein a novel two stage process can be performed for the conversion of keratin rich waste material into a useful protein rich product. Firstly, a keratin sample is used as a bacteria feed to promote the synthesis of microbial keratinases. Here, keratin consumption is not of interest and the process stage should be optimized solely for enzyme production and growth. Subsequently keratin hydrolysis may be performed using the enzyme produced in the previous stage. The two processes (enzyme synthesis and hydrolysis) are favoured in drastically different conditions, so staging is essential. The two stages can be physically separated via a cross-flow ultrafiltration membrane step, with each stage cyclically repeated in order to achieve semi-continuous operation.

It is desirable to perform the keratin degradation process stage at high solids loadings to maximize product titer and reduce process water, energy usage, and reactor size (Gong et al., 2015). As this is an industrial process in its infancy, the enzymatic hydrolysis mechanism is not well documented, rendering current industrial application limited. Therein lies a strong incentive for dynamic modelling of keratin hydrolysis, to facilitate high-fidelity process simulation and optimisation (Rodman and Gerogiorgis, 2017).

## 2. Materials and Methods

### 2.1. Enzyme preparation

Lab scale hydrolysis experiments have been performed using a keratinolytic enzymatic cocktail with the filamentous bacterium *Amycolatopsis keratiniphila* D2 (DSM 44409), first reported by Al-Musallam et al. (2003). The bacterium was cultivated on mineral keratin medium with the following composition: 0.75 g L<sup>-1</sup> NaCl, 1.75 g L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 0.25 g L<sup>-1</sup> MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.055 g L<sup>-1</sup> CaCl<sub>2</sub>, 0.010 g L<sup>-1</sup> FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.005 g L<sup>-1</sup> ZnSO<sub>4</sub>·7H<sub>2</sub>O and 1% w/w poultry by-product meal (PBM) keratin powder. The medium is sterilized at 121 °C (20 min), and free keratinase extract is obtained for hydrolysis.

### 2.2. Keratinase activity screening

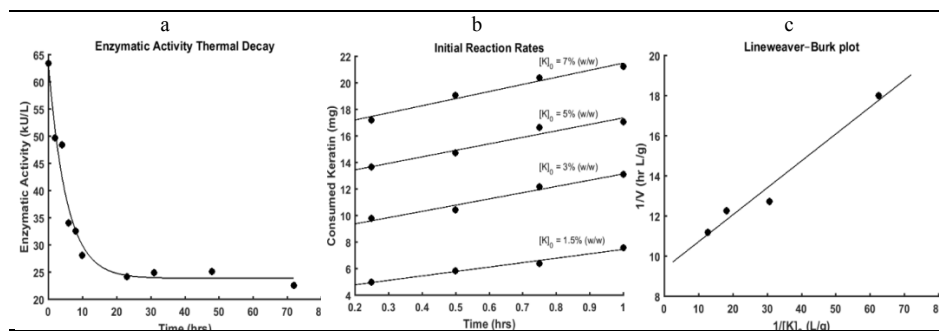
To screen for suitable reaction temperatures, enzymatic activity was monitored over time following isothermal incubation by assaying with azokeratin as a substrate. A range of temperatures were screened between 30 and 50 °C, where an inherent trade off exists between increased initial activity and increasing activity decay rate. It was found that at 40 °C the activity was suitably high and did not decay prohibitively quickly, and has thus been implemented as the experimental and model reaction temperature.

### 2.3. Dynamic state data

Vials containing 2 mL keratinase preparation and varying solids loading (PBM meal) were placed in a thermoshaker at 40 °C and 600 rpm. At fixed time intervals, triplicate vials were removed for each solid loading considered. A sample from each vial was taken and the protein content determined by bicinchoninic acid (BCA) assay, and the remaining vial contents vacuum filtered, dried and weighed to determine residual substrate mass. Two campaigns were performed: firstly, initial reaction kinetics for four substrate concentrations (1.5, 3, 5 and 7% w/w) were considered with samples taken over time in the first hour, a period in which the consumption rate is approximately constant at the maximum (initial) value. Secondly, three substrate concentrations were considered (3, 5 and 7% w/w) with the hydrolysis performed for 72 h to construct state profiles for both substrate,  $[K]$ , and product,  $[P]$ , throughout the entire reaction duration.

## 3. Experimental Results and Dynamic Model Parameter Estimation

The keratinase activity profile at 40 °C can be seen in Fig. 1a, with Fig. 1b representing the initial substrate consumption rates at differing solid loadings. The gradients from Fig. 1b are used in the Lineweaver-Burk plot (Fig. 1c) to elucidate initial reaction kinetics. Fig. 2 presents the experimental state data over a 72-hr experimental campaign.



**Figure 1.** Enzyme activity, initial reaction rates and Lineweaver-Burk plot ( $T = 40$  °C).

Based on the experimental results, the following model is proposed for the enzymatic hydrolysis of keratin. The substrate is considered to consist both hydrolysable,  $[K]_H$ , and non-readily hydrolysable,  $[K]_{NH}$ , components (Eq. 1), where the later refers to keratins which *A. keratiniphila* D2 is unable to digest. The hydrolysable fraction,  $H$ , is defined by Eq. (2). The consumption rate of the readily hydrolysable substrate is considered as the product of three factors (Eq. 4). Firstly, the Michaelis–Menten expression,  $\varphi_1$ , describes the reaction kinetics (Eq. 5). Secondly,  $\varphi_2$  relates to the keratinase activity: this term differs from a conventional two parameter first order activity decay expression by the addition of residual activity ( $R_A$ ). This is a result of an enzyme cocktail being present in place of a single cell type, where components of the cocktail have drastically differing decay timescales at this reaction temperature. The residual activity represents the activity of the cells that do not notably decay within the hydrolysis timescale, which is visible from the activity in Fig. 1a plateauing well above 0. Lastly, an inhibition term,  $\varphi_3$ , is considered as a function of the product concentration (Eq. 7), representing the proteins being produced impeding the keratin-enzyme interaction. A product ratio,  $f$ , relates protein production to substrate consumption, with the remainder of the consumed substrate mass consisting of released fats, lipids, peptides etc.

$$[K] = [K]_{NH} + [K]_H \quad (1)$$

$$[K]_H = H \cdot [K] \quad (2)$$

$$\frac{d[K]_H}{dt} = -r \quad (3)$$

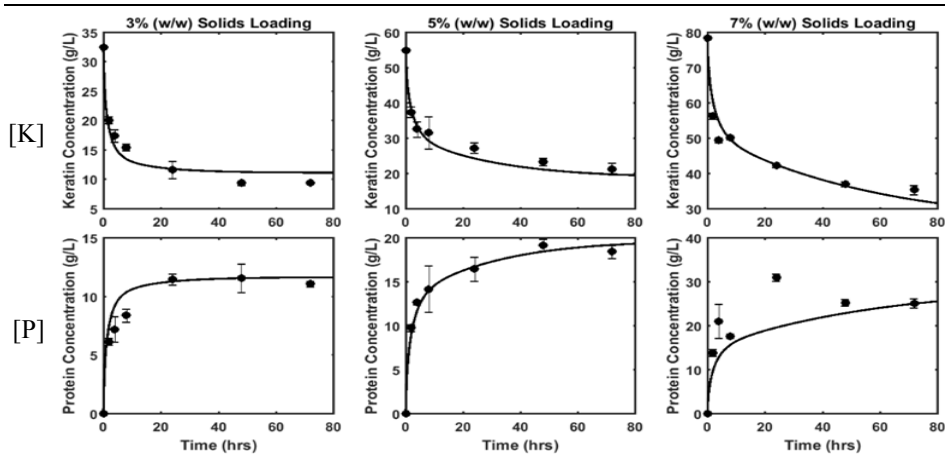
$$r = \varphi_1 \cdot \varphi_2 \cdot \varphi_3 \quad (4)$$

$$\varphi_1 = \frac{V_{max} \cdot [K]_H}{K_M + [K]_H} \quad (5)$$

$$\varphi_2 = [E] = e_i \cdot \exp(-k_D \cdot t) + R_A \quad (6)$$

$$\varphi_3 = \frac{K_I}{K_I + [P]} \quad (7)$$

$$\frac{d[P]}{dt} = f \cdot r \quad (8)$$



**Figure 2.** Dynamic model trajectories vs. experimental data for substrate and product.

**Table 1.** Dynamic model parameter values computed for enzymatic keratin hydrolysis.

| Parameter                         | Symbol    | Value | Units                            | Data Source |
|-----------------------------------|-----------|-------|----------------------------------|-------------|
| Hydrolysable substrate fraction   | $H$       | 0.63  | (-)                              | Fig. 2      |
| Maximum reaction velocity         | $V_{max}$ | 3.20  | $\text{g L}^{-1} \text{hr}^{-1}$ | Fig. 1c     |
| Michaelis–Menten constant         | $K_m$     | 14.29 | $\text{g L}^{-1}$                | Fig. 1c     |
| Initial enzymatic activity        | $e_i$     | 39.96 | $\text{kU L}^{-1}$               | Fig. 1a     |
| Enzyme decay constant             | $k_D$     | 0.188 | $\text{hr}^{-1}$                 | Fig. 1a     |
| Residual enzyme cocktail activity | $R_A$     | 23.91 | $\text{kU L}^{-1}$               | Fig. 1a     |
| Product inhibition constant       | $K_I$     | 0.328 | $\text{g L}^{-1}$                | Fig. 2      |
| Product ratio                     | $f$       | 0.548 | (-)                              | Fig. 2      |

Of the eight model parameters, three ( $e_i$ ,  $k_D$  and  $R_A$ ) can be fit from the activity assay profile (Fig. 1a), whose solid line shows the fit using parameter values from Table 1. Two parameters ( $V_{max}$  and  $K_m$ ) are determined directly using the initial kinetics via the Lineweaver-Burk plot method (Fig. 1c). Additionally,  $f$  can be directly inferred as the ratio between the protein and keratin state derivatives in Fig. 2, while  $H$  can be considered as the average of the total substrate fraction digested after the reaction is completed. Subsequently, the remaining model parameter,  $K_I$ , is estimated by minimizing the sum squared error between model and experimental data points, defined by Eq. 9 and solved using MATLAB (fminsearch); ode45 is used for model integration.

$$\min \sum (f(\mathbf{x}_j, \theta) - y_j)^2 \quad (9)$$

Here,  $f(\mathbf{x}_j, \theta)$  is the model predicted keratin and protein state trajectory for experiment  $j$ ,  $\theta$  is the parameter vector and  $y_j$  is the experimental state trajectory. The data sets for 3% and 7% w/w initial substrate concentration were used in the fitting, leaving the 5% w/w profile as a supplementary dataset which is used for comparisons for model validation.

#### 4. Discussion

It can be seen from Fig. 2 that the proposed model is able to effectively describe the key behaviour observed in the experimental data, both for keratin consumption and protein production. The dataset not used in the model parameter determination (5% w/w loading) shows good agreement between experimental data and the model prediction, suggesting the model can accurately describe keratin hydrolysis at 40 °C with *A. keratiniphila* D2. The model can be used to compute optimal substrate dosage strategies towards processing time and cost minimization in biochemical waste keratin hydrolysis.

The model assumption of a fixed fraction of the substrate being hydrolysable,  $H$ , is not able to fully capture the observed phenomena where yield decreases with solids loading. As a result, the model under-predicts keratin consumption at 3% w/w solids, and over predicts for 5% and 7% w/w (Fig. 2). If the value of  $H$  is defined individually for each solid loading campaign according to the observed experimental yield in that campaign and the model parameters re-determined, the fit is exceptional. This indicates that if the mechanism behind diminishing yield with increasing substrate concentration can be incorporated into an updated model it would be even more accurate in representing the dynamic system. It is known that as solids loading increases, factors that were insignificant in low-solid systems become more prominent (Modenbach and Nokes, 2013), which can restrict substrate conversion yield at higher loading and is found to be the case here. It is possible that mass transfer between the keratin and enzyme is becoming impeded at high substrate concentrations due to reduced free water content as the liquid absorbs into the biomass, as has been observed in high-solids enzymatic

cellulose hydrolysis (Hodge et al., 2009), a reactive system known to have many parallels to keratin hydrolysis; however further experimental work is necessary to confirm whether this is the precise mechanism responsible for the observed phenomena. Moreover, further experimental work to investigate parameter temperature dependence will have great value towards process development, facilitating model implementations towards simultaneous optimization of reactor temperature profile and keratin dosing.

## 5. Conclusions

The hydrolysis of keratin rich material with keratinolytic bacteria is an attractive way of transforming undesirable waste from agroindustrial activities into products of practical industrial value. A lab-scale experimental campaign has been performed, enabling the successful parameterization of a dynamic model for enzymatic hydrolysis of keratin. Michaelis–Menten kinetics with product inhibition allows the observed behaviour of the reactive system to be captured, with the model fit showing good agreement with experimental data. The development of such a model is an important contribution towards improving the competitiveness of this novel means of waste valorization via computational simulation, optimization and development of hydrolysis reactors. The model assumption of a fixed fraction of the substrate being hydrolysable cannot fully capture the observed phenomena, inasmuch as yield decreases with solids loading. Further experimental work is necessary to explore this advance, with the aim of better describing the apparent inhibition effect at higher substrate content, thus increasing model fidelity and enabling dynamic optimization of the keratin hydrolysis reactor.

## 6. Acknowledgements

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